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## INTERACTION OF ISOFLAVONES AND ENDOPHYTE-INFECTED TALL FESCUE SEED EXTRACT ON VASOACTIVITY OF BOVINE MESENTERIC VASCULATURE

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INTERACTION OF ISOFLAVONES AND ENDOPHYTE-INFECTED TALL FESCUE  
SEED EXTRACT ON VASOACTIVITY OF BOVINE MESENTERIC  
VASCULATURE

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THESIS

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A thesis submitted in partial fulfillment of the  
requirements for the degree of Master of Science in the  
College of Agriculture, Food and Environment  
at the University of Kentucky

By  
Yang Jia  
Lexington, Kentucky

Director: Dr. D.L. Harmon, Professor of Animal Science  
Lexington, Kentucky  
2014

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## ABSTRACT OF THESIS

### INTERACTION OF ISOFLAVONES AND ENDOPHYTE-INFECTED TALL FESCUE SEED EXTRACT ON VASOACTIVITY OF BOVINE MESENTERIC VASCULATURE

Endophyte-infected tall fescue produces many ergot alkaloids, which have been shown to be vasoconstrictive in various vessel types of bovine. On the other hand, substantial evidence has been reported on the vasodilative effects of formononetin and biochanin A in different vessel types in humans and rats. So, a study was conducted using mesenteric vasculature collected from heifers shortly after slaughter. After 2-h incubation with formononetin (F), biochanin A (B), or an ergovaline-containing tall fescue seed extract (EXT) and their combinations, vessels were mounted in a multi-myograph to determine their ergotamine-induced contractility. Results indicated that F and B at  $1 \times 10^{-6} M$  and their combination did not impact the contractile response to ergotamine in mesenteric vasculature. The pre-myograph incubation of mesenteric vasculature with EXT altered the contractile response manner to ergotamine. However, at higher concentration, F and B may alleviate the reduction of vasoconstriction caused by prior exposure to EXT. To our knowledge, this study was the first to investigate the interaction of ergot alkaloids and isoflavones on in vitro bovine mesenteric vasculature. However, further investigations are necessary to understand the mechanism behind the interaction of ergot alkaloids and isoflavones on vasoactivity.

KEYWORDS: Bovine; Ergot alkaloids; Isoflavones; Mesenteric Vasculature;

Vasoconstriction

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December 9, 2014

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SEED EXTRACT ON VASOACTIVITY OF BOVINE MESENTERIC  
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## Chapter 1. Literature Review

### Ergot Alkaloids

#### History

Ergot alkaloids have been playing important roles, as toxins and natural pharmaceuticals, throughout human history. It has been speculated that ergot alkaloids which induced hallucinations were the culprit four thousand years ago in the Eleusinian Mysteries of ancient Greece (Wasson et al., 1978). Although used in obstetrics as early as 1100 B.C. for their medicinal effects (Schiff, 2006), poor understandings of their physiological effects and toxicodynamics limited the beneficial uses of ergot alkaloids in these early times.

During the Middle Ages, several bizarre epidemics outbreaked in various medieval European countries and killed thousands of people (Wasson et al., 1978; Schiff, 2006). Those outbreaks are now understood and have been attributed to long-term ergot poisoning, also termed as ergotism (Caporael, 1976). The cause of ergotism is indirect ingestion of ergot alkaloids from ergot-contaminated grains (Strickland et al., 2011). Two different forms of toxic symptoms were characterized from these ergotism outbreaks. The first form was described as gangrenous form (*Ergotismus gangraenosus*) (Schiff, 2006), also referred to as “Holy fire”, or “St. Anthony’s fire”, which vividly portrays the severe burning pain (“fire”) in the extremities (Wasson et al., 1978; Flieger et al., 1997; Schiff, 2006). It is now obvious that prominent peripheral vasoconstriction initiated the

swelling and extreme burning pain of limbs, and eventually caused gangrene (Flieger et al., 1997).

The second type of symptoms was called the convulsive form (Ergotismus convulsivus) (Schiff, 2006). Persons with this type developed delirium and hallucinations in Eleusinian Mysteries of ancient Greece as Wasson et al. (1978) speculated. However, recent incidence of ergotism has been very scarce and results from the achievements in agriculture in terms of genetics and management and on techniques of grain sanitization which removes most of the sclerotia (Flieger et al., 1997).

With improved understanding of ergot alkaloids, their beneficial uses have been discovered. Examples of their pharmacological effects include: cerebrovascular insufficiency, prolactin inhibition, treatment of Parkinsonism, thrombosis, stimulation of cerebral and peripheral metabolism, venous insufficiency, uterine stimulation, and as a dopaminergic agonist (Aellig et al., 1978).

The first ergot alkaloids (ergotoxine, which later found to be a mixture of four alkaloids) were isolated by G. Barger and H. Dale in 1906 (De Costa, 2002). Ergotamine, as the first pure alkaloid, was isolated by Arthur Stoll in 1918 (Peroutka, 1996). Isolated pure ergot alkaloids allowed researchers to have a closer look at their structures and chemistry, which is highly beneficial for understanding and further exploitation of their medical benefits. Nowadays, researchers are focusing on molecular, biological and biochemical investigations in terms of ergot alkaloid biosynthesis (Wallwey and Li, 2011).

## Chemistry of Ergot Alkaloids

So far, approximately 50 natural ergot alkaloids have been characterized (Flieger et al., 1997). The common structural feature of all ergot alkaloids is a tetracyclic ergoline ring system (Figure 1.1) (Garner et al., 1993; Flieger et al., 1997; Schiff, 2006; Strickland et al., 2011), with nitrogen methylated at the 6 position and carbon substituted variously at 8 position. From a biosynthetic perspective, the indole moiety is derived from tryptophan (Bu'Lock and Barr, 1968). Acetate is metabolized to dimethylallylpyrophosphate, which is found in many biological systems (Garner et al., 1993). In many ergot alkaloids, a double bond usually exists between C-8 and C-9 or C-9 and C-10. The position of the double bond permits compounds to have epimers, which is reported to not only have different sensitivities to temperature and solvents (Hafner et al., 2008), but also differing biological potency in animal systems (Berde and Stürmer, 1978; Berde, 1980). Based on the different type of substitutes on C-8 of ergoline ring, ergot alkaloids are classified into three groups: 1) clavine alkaloids, 2) lysergic acid and its simple amides (derivatives), 3) ergopeptine alkaloids (Figure 1.2) (Lyons et al., 1986; Porter, 1995; Schardl et al., 2006; Wallwey and Li, 2011). As shown in Figure 1.2, clavine alkaloids generally are 6, 8-dimethylergolines with a few exceptions, such as chanoclavine, which has a 6, 7-Seco ring. Lysergic acid is characterized as a substituted carboxyl group on C-8 of ergoline ring. Amidation of the carboxyl group on C-8 derives the lysergic acid amide (Schiff, 2006). Due to their high pharmacological activity, lysergic acid amide, such as ergometrine and methylergometrine, have been used to prevent and treat postpartum hemorrhage (De Costa, 2002; Rajan and Wing, 2010).

The third group, ergopeptine alkaloids, are structurally characterized as lysergic acid derivatives with a tripeptide moiety attached to its carboxy group by peptide-like amide bonds (Figure 1.3) (Wallwey and Li, 2011). Various substitutions at R1 and R2 accounts for the diversity of ergopeptine alkaloids (Table 1.1).

Among ergopeptines, ergovaline and ergotamine commonly draw more attention of researchers. Lyons et al. (1986) reported that ergovaline was the predominant (84 to 97%) alkaloids of all the five detected ergopeptine alkaloids from tall fescue (*Lolium arundinaceum*) pasture. Both ergovaline and ergotamine are vasoconstrictive and therefore have some therapeutically significant properties, especially ergotamine and its derivatives which have been used as a therapy for vascular headaches, such as migraines (Schiff, 2006). The structural similarities of ergoline ring to the biogenic amines, dopamine, epinephrine, norepinephrine, serotonin, explains many of the biological effects of ergot alkaloids at the cellular or molecular level (Weber, 1980). The “mistaken” recognition and binding by biogenic amine receptors renders ergot alkaloids active in some of the biological agonist roles. This is the pharmacological rationale of ergot alkaloids as they are applied to treat rhage, migraine, venostasis, hyperprolactinemia and Parkinson’s disease (Berde, 1980).

#### Natural Sources of Ergot Alkaloids

Naturally, ergot alkaloids are produced by fungal endophytes, mainly within the *Hypocreales* and *Eurotiales* orders. *Claviceps* genus under *Hypocreales* order have been known and parasitize more than 600 plants (Strickland et al., 2011). *Claviceps sclerotia*

were also found in several important grains such as barley, wheat, rye, millet, and oats (Bové, 1970; Groger, 1972), which was the major culprit of the ergotism outbreaks during the Middle Ages in Europe.

Since Stoll first isolated and identified ergotamine from *Claviceps sclerotia* (Stoll, 1952), several other endophytic fungi genus such as *Epichloë*, *Neotyphodium*, and *Balansia*, have been proven to produce ergot alkaloids on contaminated plants (Bacon, 1988; Flieger et al., 1997; Clay and Schardl, 2002). However, the production of ergot alkaloids varies depending on the interactions of different fungi and plants. For example, fungi *Aspergillus* and *Penicillium* possess a lower capability of producing ergot alkaloids than *Claviceps* (Reshetilova and Kozlovskii, 1990; Flieger et al., 1997).

On the other hand, ergot alkaloids are distributed differently within the plant. For instance, ergot alkaloid concentrations in tall fescue from high to low is generally seed, crown, stems, leaves, and roots (Rottinghaus et al., 1991; Azevedo and Welty, 1995). What's more, environmental factors (growth temperature, humidity, soil fertility) also play big roles in impacting the growth of certain fungi and further regulating the production of ergot alkaloids. The symbiotic interactions between plants and endophytic fungi benefit the host in terms of the greater resistance to insects, herbivores, pathogens, as well as enhanced environmental tolerance and overall competitiveness. Even though the endophyte infected grains are no longer an ergotism risk for humans, the ergot alkaloids produced by these endophytes, especially *Neotyphodium coenophialum*-infected tall fescue (*L. arundinaceum*), hamper livestock production (Strickland et al., 2011).

## Fescue Toxicosis and Its Influence on Livestock

Tall fescue [*Lolium arundinaceum* (Schreb.) Darbysh.] is the most widely distributed cool-season perennial pasture grass in the world. Although tall fescue is a native of Europe, it has been distributed and cultivated in North and South America, southern Africa, Australia, New Zealand, and eastern Asia (Hannaway et al., 2009). Tall fescue is the predominant temperate pasture grass grown within the transition zone of the eastern and central part of the United States (Strickland et al., 2009a), covering approximately 15 million ha (Buckner et al., 1979).

With the wider occurrence and application of tall fescue in pastures, there were reports of grazing animal having health problem and poor performance (Pratt and Haynes, 1950; Pratt and Davis, 1954). Following this, several grazing studies found gains of beef steers on tall fescue were significantly lower than on orchardgrass (*Dactylis glomerata* L.) (Blaser et al., 1956; Mott et al., 1971; Harris, 1972). Other than decreased bodyweight gains and cow conception rates (Petritz et al., 1980), three major disorders have been observed and characterized (Stuedemann and Hoveland, 1988).

*Fescue foot* is considered the most dramatic visible symptom which was first reported by Cunningham (1949) in New Zealand. The incidence was usually higher in cold weather with the signs: stiffness and soreness of tissue (dry gangrene) appearing near the end of hoof or tail, lameness in hind quarters, loss of tail switch (Cunningham, 1949). These disorders are similar with the symptoms of gangrenous form ergotism, which is caused by the vasoconstrictive effect of ergot alkaloids on extremities.



The other two symptoms, *summer slump* and *fat necrosis*, have been intensively reviewed (Bush and Buckner, 1973; Strickland et al., 1993; Strickland et al., 2009a; Waller, 2009) and are characterized by reduced bodyweight gain, rough hair coat, elevated body temperature and respiration rate, reduced milk production, lesser tolerance of heat. In general, these three typical symptoms belong to a more comprehensive toxicity syndrome, termed as tall fescue toxicosis (also known as fescue toxicosis) (Strickland et al., 2009a).

The first conclusive evidence was unclear until Bacon et al. (1977) and coworkers first reported that the presence of an endophytic fungi, *Epichloë typhina*, in tall fescue which associated with fescue toxicosis. Later, this endophyte was renamed as *Acremonium coenophialum*, and then *Neotyphodium coenophialum* (Morgan-Jones and Gams, 1982). Unsurprisingly, the clavine alkaloids and lysergic acid amides (Porter et al., 1981; Yates and Powell, 1988; Yates et al., 1989; Petroski and Powell, 1991), and ergopeptine alkaloids (Yates et al., 1985; Lyons et al., 1986; Yates and Powell, 1988) were all detected from *Neotyphodium coenophialum*-infected tall fescue.

In combination with the symptom similarity of fescue toxicosis and ergotism, ergot alkaloids are most likely the causative agents of fescue toxicosis. As proved later by many researchers, ungulates' consumption of ergot alkaloids produced by *Neotyphodium coenophialum*-infected tall fescue induces the fescue toxicosis, which is considered as the main grass-induced toxicosis in the United States (Cheeke, 1995). Considering tall fescue's prevalence as pasture grass in the southeastern United States, it is self-evident that the economic loss on livestock production of fescue toxicosis is profound. In United States,

the annual economic losses from decreased conception rates and weaning weights of beef cattle grazing endophyte-infected tall fescue were conservatively estimated at \$609 million (Hoveland, 1993). When taking into account the influences on the equine and small ruminant industries, as well as calculations based on today's dollars, the overall annual losses are estimated to exceed 1 billion dollars (Strickland et al., 2011). Admittedly, a comprehensive solution to fescue toxicosis has not been proposed so far, neither as a systematic mechanism of ergot alkaloids on different physiology systems of domestic animals. Nevertheless, a great deal of research has been and will be conducted to better understand the toxicodynamics of fescue toxicosis and to further explain the complex interaction among the animal, ergot alkaloids, plant, endophyte and environmental factors involved (Strickland et al., 2011).

### Toxicodynamics

Grazing endophyte-infected tall fescue induces several disorders of different physiological systems (gastrointestinal, reproduction, cardiovascular, etc.) (Strickland et al., 2011) which are summarized in general as fescue toxicosis. In terms of seeking more comprehensive and effective solutions of fescue toxicosis, it is vital to thoroughly understand the physiological mechanisms behind every fescue toxicosis induced disorder. It has been intensively reviewed in regard to the impact of ergot alkaloids from toxic endophyte-infected tall fescue on the animals microbial ecology, gastrointestinal system, neural and neuroendocrine systems, reproductive system, immune system, cardiovascular system, and the possible toxicodynamics associated with them (Strickland et al., 2009a; Strickland et al., 2011). In this section, the major focus will be located on the

ergot alkaloids' impact on cardiovascular system, especially on core or peripheral blood vascular function and the potential mechanism behind them.

As mentioned previously, grazing endophyte-infected tall fescue results in major impacts on vascular function, which leads to the symptoms of gangrene of extremities and elevated body temperature. In support, research findings tend to favor ergot alkaloids as the primary causative agents of fescue toxicosis (Solomons et al., 1989; Oliver et al., 1992; Oliver et al., 1993; Strickland et al., 1996; Oliver et al., 1998; Klotz et al., 2006; Klotz et al., 2007), especially in consideration of their biological activities on blood vessels. Two primary effects of ergot alkaloids on cardiovascular systems of domestic animals have been summarized (Strickland et al., 2011). First, immediate reduction of heart rate appears at 4 hours after E+ tall fescue consumption, and gradually returns back to pre-exposure level in about 172 hours (McLeay et al., 2002; Aiken et al., 2007; Aiken et al., 2009). Second, vasoconstriction and possible morphological changes (such as smooth muscle cell hyperplasia and endothelial cell damage) of blood vessels (Rhodes et al., 1991; Strickland et al., 1996; Oliver and Schultze, 1997; Shappell, 2003; Aiken et al., 2007; Aiken et al., 2009). This section will mainly focus on the effects of ergot alkaloids on vascular function (i.e., vasoconstrictive effects, changes of smooth muscle cell and endothelial cell), and the mechanisms currently thought to be involved.

#### Effects on Vascular Smooth Muscle

Vascular smooth muscle is the middle of the three concentric layers of blood vessels, and is important in regulating the caliber of the blood vessels by contraction or

relaxation (Strickland et al., 2009b). Endothelium lines the interior surface of blood vessels and regulates vascular homeostasis by interacting with a variety of paracrine factors (Vita and Keaney, 2002). Symptoms of fescue foot were first induced in calves given ethanolic extracts of tall fescue hay (Williams et al., 1975). They also reported that calves that had fescue foot had concomitant thickening of vessel walls and smaller vessel lumens in blood vessels of the coronary bands and tail tips. Garner and Cornell (1978) reported similar observations with a thickening of the smooth muscle layer of peripheral blood vessels after consumption of endophyte-infected tall fescue. However, it was unclear whether the growth of smooth muscle cells is based on a mechanism of hyperplasia or hypertrophy. Later, the findings of Strickland et al. (1996) appear to favor hyperplasia over hypertrophy as a possible mechanism. They reported that ergonovine, ergovaline and  $\alpha$ -ergocryptine stimulate the growth of quiescent bovine vascular smooth muscle cells in vitro. However, inhibitory effects of bromocriptine (a semisynthetic derivative of peptide alkaloids) on growing vascular smooth muscle cell proliferation was observed in vitro in the rat and human (Zhang and Cincotta, 1997). Similarly, ergovaline inhibited the growth of growing bovine vascular smooth muscle cells (Strickland et al. (1996).

#### Effects on Vascular Endothelium

The endothelium is also highly suspected to be impacted by ergot alkaloids (Strickland et al., 2009b). There was a debate based on whether ergot alkaloids have direct influence on endothelial cells or not. One argument was that instead of toxic effects of ergot alkaloids (ergotamine), blood vasoconstriction caused endothelial damage (Lewis

and Gelfand, 1935). These workers found that a necrosis was induced in the fowl's comb by ergotamine and further concluded that vasoconstriction alone did not necessarily cause gangrene until the endothelial damage appears as a consequence of prolonged vasoconstriction. Their point of view was supported by Thompson et al. (1950) who reported a migrainous case subject with non-gangrenous arteriospasm with no organic changes in the small vessels. On the other hand, ergot alkaloids were reported to induce bovine dorsal pedal vein endothelial cell death in cultures (Shappell, 2003). In consideration of the concentration of ergot alkaloids used in this study was relatively higher than natural grazing scenarios and further research is needed to support Shappell's argument.

#### Vasoconstrictive Effects

Regardless of the contradictory findings in vascular smooth muscle cell and endothelial cell, ergot alkaloids have produced vasoconstrictive effects in a variety of in vivo and in vitro research. Due to the intimate relationship of fescue toxicosis symptoms (e.g., gangrene in extremities and elevated body temperatures) and peripheral vasoconstriction, caudal artery (Aiken et al., 2007; Aiken et al., 2009), dorsal pedal vein (Solomons et al., 1989) and lateral saphenous vein (Klotz et al., 2006) have been used by several studies as a peripheral blood vessel model. Other models [e.g., right ruminal artery and vein (Klotz et al., 2011), mesenteric artery and vein (Klotz, 2014)] have also been developed in order to investigate the effects of ergot alkaloids on core blood vessels and their interaction with nutrient absorption.

However, there is only one published paper focusing on the effects of whole body vasculature by ergot alkaloids in sheep and cattle (Rhodes et al., 1991). Using radiolabeled microspheres, they reported that reduced ( $P < 0.10$ ) blood flow occurred lower in hind leg skin and adrenal glands by wethers fed a high alkaloid (1.18 ppm ergovaline) diet. Similar blood flow reductions to the rib skin ( $P = 0.002$ ), duodenum ( $P = 0.084$ ), colon ( $P = 0.080$ ) and kidney ( $P = 0.118$ ) were reported in cattle fed a high endophyte (0.52 ppm ergovaline) diet under high ambient temperatures (32°C.) (Rhodes et al., 1991). These findings might explain the higher rectal temperature of animals exposed to endophyte-infested tall fescue. The constricted blood vessel reduces its vessel caliber and therefore limits the blood flow to peripheral tissues, which results in inefficiency of body heat dissipation from core body to the surface (Strickland et al., 2009a).

Several other in vivo studies have been conducted to investigate the vasoconstrictive effects of ergot alkaloids on peripheral blood vessel models. A modified mercury-in-rubber strain gauge and plethysmographic from human limb model (Whitney, 1953) was used to measure the blood flow in the tail of dairy Heifers (Walls and Jacobson, 1970). They reported that heifers treated with an alcoholic extract of fescue hay (likely contains ergot alkaloids) showed a decreased blood flow in the tail. Aiken and colleagues conducted two experiments to investigate the hemodynamic responses of the caudal artery by using color Doppler ultrasonography (Aiken et al., 2007; Aiken et al., 2009). They found a decreased caudal artery area and blood flow after heifers exposed to endophyte infested tall fescue.

An in vivo trial was designed to evaluate the impact of ergot alkaloids on reticuloruminal epithelial blood flow at thermoneutral (22 °C) and heat stress (32 °C) (Foote et al., 2013). The epithelial blood flow was measured as ruminal clearance of D<sub>2</sub>O corrected for influx of physiological water and liquid outflow. They reported that ergot alkaloids from an endophyte infected tall fescue seed extract (0.015 mg ergovaline · kg BW<sup>-1</sup>·d<sup>-1</sup>) caused at least a 50% reduction in epithelial blood flow of steers regardless of temperature. In addition, a reduced VFA absorption was observed and suspected to be associated with the reduction in blood flow to the absorptive surface of the foregut (Foote et al., 2013).

A series of bioassays have been developed to address the effects of ergot alkaloids on blood vascular functions in livestock. This approach allows researchers to investigate the effects of individual ergot alkaloid on certain target blood vessels, while in vivo trials are extremely difficult to create these specific combinations (Strickland et al., 2009b). Therefore, this methodology is not only suitable to studies looking at detailed dose response of certain ergot alkaloids and blood vessel combinations, but also is able to evaluate and compare the vasoconstrictive potency and efficacy of individual ergot alkaloids. What's more, the direct dose of ergot alkaloids to target blood vessels in vitro will eliminate the possible interference that may be induced from the interactions of ergot alkaloids with other physiological systems. As mentioned in the previous section, the differences in ergot alkaloids on chemical structure and abundance in endophyte infested tall fescue has raised the question of which single ergot alkaloid contributes more to the vasoconstriction? In other words, the potency and efficacy have become the

interest of many studies, in terms of understanding the pharmacological mechanism of ergot alkaloid on vasculature.

An in vitro bioassay based on bovine dorsal pedal vein was developed by Solomons et al. (1989) and reported that ergotamine, agroclavine and ergosine were able to elicit constriction of bovine dorsal pedal vein. Ergotamine induced the highest maximal contractile force, as well as required the lowest concentration to induce a constrictive response. A series of studies, using a similar bioassay, have been conducted to investigate the potency and efficacy of certain ergot alkaloids on bovine lateral saphenous vein (model of peripheral vessels) (Klotz et al., 2006; 2007; 2008, 2010; Pesqueira et al., 2014). Ergotamine and ergovaline produced similar contractile responses on saphenous veins in terms of initial contractile responses (both at  $1 \times 10^{-8}$  M) and maximum contractile tension (Klotz et al., 2007). However, lysergic acid was reported to induce an appreciable contractile response at  $1 \times 10^{-4}$  M, and where maximum contractile force was achieved ( $15.6 \pm 2.3\%$  of norepinephrine response) (Klotz et al., 2006). Based on these, it is reasonable to say ergotamine and ergovaline are more toxic to bovine lateral saphenous vein than lysergic acid, with at least 1000-fold more potency and 5-fold more efficacy (Strickland et al., 2011).

Other ergot alkaloids, ergonovine,  $\alpha$ -ergocryptine, ergocristine, and ergocornine induced contractile responses on the saphenous vein, and potency and efficacy were intermediate to ergovaline and lysergic acid (Klotz et al., 2010). The structural differences among ergot alkaloids were thought to impact their affinity or mode of binding to



serotonin receptors (Choudhary et al., 1995), which could result in different contractile response and potency.

Other than peripheral vessel models (i.e., dorsal pedal vein, lateral saphenous vein), this bioassay have been applied on core vessels such as the right ruminal artery and vein (Foote et al., 2011; Klotz et al., 2011), and mesentery artery and vein (Egert et al., 2014) to determine the effects of ergot alkaloids on vascular function and further interactions with gut physiology. Derived from their previous peripheral lateral saphenous vein model (Klotz et al., 2006), Klotz et al. (2011) developed and validated a vascular contractility bioassay using bovine right ruminal artery and vein, to determine the vasoconstrictive potentials of ergovaline, ergotamine, ergocryptine, ergocristine, ergonovine, ergocornine and lysergic acid (Foote et al., 2011). They reported, in both vessel types, lysergic acid failed to induce a contractile response, which was contradicted to the findings that lysergic acid is vasoconstrictive on the lateral saphenous vein (Klotz et al., 2006; Klotz et al., 2008). However, the appreciable contractile response was not observed until lysergic acid at  $10^{-4}$  M, which is not an achievable concentration for natural grazing animals. Foote et al. (2011) also found ergotamine and ergovaline were potent vasoconstrictors with relatively lower  $EC_{50}$  (indicator of potency) and higher  $E_{max}$  (indicator of efficacy) than other ergot alkaloids in both ruminal arteries and veins, which was in agreement with previous saphenous vein studies.

It may not be appropriate to compare ergot alkaloid's potency and maximum contractile force in an absolute magnitude between this (Foote et al., 2011) and previous saphenous vein studies (Klotz et al., 2006; Klotz et al., 2007; Klotz et al., 2008, 2010), since

there are differences in several vital setups between the two bioassays (e.g., Instead of norepinephrine, 0.12 M KCl was used as a reference compound in ruminal artery and vein model. (Klotz et al., 2011)). Nevertheless, taken together, these findings demonstrated that both ergotamine and ergovaline are potent vasoconstrictors, while lysergic acid is unable to cause vasoconstrictions of bovine tissues under normal physiological conditions.

Based on available data (Hill et al., 2001; Ayers et al., 2009), the small intestine may be the major absorption site of ergopeptine alkaloids (Strickland et al., 2011). What's more, the mesenteric vein is one of the two outlets of transporting ergot alkaloids after they cross the gastrointestinal epithelia, which leads to portal vein and liver (Eckert et al., 1978). Thus, a bioassay was derived from the previous right ruminal artery and vein model (Klotz et al., 2011) to profile the vasoactivity of ergot alkaloids in the bovine mesenteric artery and vein (Egert et al., 2014). Similarly, ergocryptine, ergotamine, ergocristine, ergocornine, ergonovine, and ergovaline-containing tall fescue seed extract, and serotonin were all observed to induce a contractile response on mesenteric artery and vein, with the exception of lysergic acid. They also reported that steers with previous dietary exposure to endophyte-infested tall fescue seed had decreased or lacked a constrictive response to many ergot alkaloids in the small intestine vasculature (Egert et al., 2014), which were consistent with the findings in lateral saphenous vein (Klotz et al., 2012; Klotz et al., 2013).

## Mechanisms

Given the studies above, ergot alkaloids associated with endophyte infested tall fescue are able to induce vascular contraction in multiple tissues of different animal models. Therefore, they have been attributed as the major causative agents of fescue toxicosis. The core ergoline ring system of ergot alkaloids shares some structural similarities with several biogenic amines (i.e., (nor)epinephrine, serotonin, and dopamine) (Weber, 1980), which may confer to ergot alkaloids the ability to interact with corresponding biogenic amine receptors (Berde, 1980). Several pharmacological researchers have provided evidence of  $\alpha$ -adrenergic receptors that are associated with ergot alkaloids and blood vessel interactions (Byrne-Quinn, 1964; Fedotin and Hartman, 1970; Greene et al., 1977). Although the exact mechanisms behind them are not fully defined, several in vitro vascular studies in livestock have reported that ergot alkaloids induced vasoactivity is mediated via several biogenic amine receptors (Table 1.2).

One of the first reports of ergot alkaloids interacting with serotonergic receptors was demonstrated by Dyer (1993). He reported that ergovaline induced contractions of bovine uterine and umbilical arteries were blocked by ketanserin and phenoxybenzamine (both 5HT<sub>2A</sub> antagonist) but not by prazosin or phentolamine (both  $\alpha_1$ -adrenergic antagonist), which indicated 5HT<sub>2A</sub> receptors but not  $\alpha_1$ -adrenergic receptors were involved in the vasoconstriction. In contrast, Schoning et al. (2001) found that the ergovaline induced rat thoracic aorta contractile response was via  $\alpha_1$ -adrenergic receptors. They also reported that ergovaline and ergotamine are equally effective as a partial agonist in rat tail artery, and their vasoconstrictive responses were antagonized by

ketanserin (5HT<sub>2A</sub> antagonist), which was in agreement with Dyer's findings. What's more, these two studies both indicated that the binding of ergovaline and 5HT<sub>2A</sub> serotonergic receptor was irreversible, and the dissociation from the receptor was slow (Dyer, 1993; Schoning et al., 2001). In the latter study, ergovaline also induced contractile responses in guinea pig iliac artery via the 5HT<sub>1B/1D</sub> serotonergic receptor, and interestingly, it acted as an agonist and (or) antagonist at the 5HT<sub>1B/1D</sub> receptor. However, Oliver et al. (1993) demonstrated that lysergamide (ergine) was a partial agonist or antagonist at  $\alpha_1$ -adrenergic and  $\alpha_2$ -adrenergic receptors, respectively, in a bovine lateral saphenous vein model. Likewise, lysergamide could induce contraction of saphenous vein via 5HT<sub>2A</sub> receptors but not 5HT<sub>1</sub> receptors (Oliver et al., 1993). Using the same bovine lateral saphenous vein model, Klotz et al. (2012) found DOI (5HT<sub>2A</sub> receptor agonist) induced vessel contractile intensities were 35% lower ( $P < 0.05$ ) in high endophyte-infested tall fescue than in low endophyte-infested tall fescue, whereas 5CT (5HT<sub>7</sub> receptor agonist) produced greater (37%,  $P < 0.05$ ) contractile intensities in high endophyte-infested tall fescue. Therefore, they indicated that chronic exposure to ergot alkaloids through grazing endophyte-infested tall fescue altered the vasoconstriction via serotonergic receptors, but in different manners. In a subsequent study (Klotz et al., 2013) they reported ketanserin (5HT<sub>2A</sub> receptor antagonist) reduced the contractile response to ergovaline, ergotamine, and ergocornine, but SB-269970 (5HT<sub>7</sub> receptor antagonist) was ineffective at altering the contractile response.

In general (Table 2), there is evidence that ergot alkaloids interact with adrenergic and serotonergic receptors as partial agonists or antagonists. The interaction between

specific receptor subtypes and ergot alkaloids varies among tissue types, as well as animal models. Future studies are necessary to better understand the mechanism of ergot alkaloid induced vasoconstriction at the receptor level, which is important to researchers in terms of discovering therapeutic solutions for fescue toxicosis.

## Isoflavones

### Introduction

Phytoestrogens are broadly defined as a group of naturally occurring compounds in plants that can exert estrogenic activities (Setchell, 1998). Usually these plant-derived nonsteroidal molecules execute more “important” functions in animals than in plants, where they mainly function as antioxidants (Anderson and Garner, 1997). The most conspicuous character of the phytoestrogens is a diphenolic ring with structural similarities of mammalian estrogens (Setchell and Adlercreutz, 1988). It is not surprising that phytoestrogens bind to estrogen receptors and act as estrogen agonist or antagonist (Shemesh et al., 1972; Verdeal et al., 1980; Barnes and Peterson, 1995; Makela et al., 1995a; Makela et al., 1995b). This interaction with estrogen receptors offers the possibility of phytoestrogens being potential alternatives therapies for several hormone dependent conditions, such as cancer, menopausal symptoms, cardiovascular disease and osteoporosis (Vitale et al., 2013). The abundant natural sources of phytoestrogens has been discovered with the development of screening and identification technologies. Although phytoestrogens are restrictedly distributed in the plant kingdom, more than 300

plants have been identified so far to induce estrogenic responses in animals (Bradbury and White, 1954; Farnsworth et al., 1975; Whitten and Patisaul, 2001).

There are three major classes of phytoestrogens, isoflavones, coumestans, and lignans (Kurzer and Xu, 1997). These have always been the interest of studies from a nutritional and health-promoting perspective. Isoflavones are the most commonly studied form of phytoestrogens, and are almost exclusively found in the legume (*Leguminosae/Fabaceae*) family (Franke et al., 1994; Reinli and Block, 1996). Soybeans and soy products are the major source of dietary isoflavones, and contain genistein, daidzein, and glycitein (Murphy, 1982; Coward et al., 1993; Wang and Murphy, 1994). Many clinical studies in humans, animals, and cell culture systems have addressed these two compounds in terms of investigating the potential health benefits of soy and soy products. Other than the soybean, chick peas, red clover, toothed medic, and bluegrass are all isoflavone containing sources (Price and Fenwick, 1985). Compared with soybeans, red clover contains more varieties of isoflavones, such as genistein, daidzein, biochanin A and formononetin (Beck et al., 2005). Structurally, biochanin A and formononetin are the 4'-methyl ethers of genistein and daidzein, respectively (Figure 4) (Kurzer and Xu, 1997).

#### Isoflavones and Impacts on Human and Animal Health

Long term hormone therapies on postmenopausal women have been reported to induce some unexpected side-effects, such as increased risks of breast cancer (Investigators, 2002; Beral et al., 2003; Anderson et al., 2004). Thus, finding an alternative compound to substitute conventional steroid hormone would be desirable.

Phytoestrogens, especially isoflavones, with weak estrogenic action, may be a potential candidate. However, substantial evidence has been provided by a variety of clinical human trials, animal and cell/tissue culture studies to indicate that isoflavones have different beneficial effects on humans in many different ways, such as cardiovascular system, osteoporosis, breast and prostate cancer (Anderson and Garner, 1997; Kurzer and Xu, 1997; Dixon, 2004; Beck et al., 2005).

It is not the intent to review the physiological effects of isoflavones on these different systems, neither to discuss the mechanisms associated with them. In this section, the topic will be mainly focusing on the effects of isoflavones on vasculature and the possible cellular or molecular mechanisms behind them.

#### Physiological Effects of Isoflavones on Vasculature

Substantial evidence has been reported on the vasodilative effects of isoflavones and their metabolites in different vessel types in humans (Walker et al., 2001; Chin-Dusting et al., 2004) and rats (Gimenez et al., 1997; Chin-Dusting et al., 2001; Jackman et al., 2007; Wu et al., 2010). Some of the first information about isoflavone metabolite activities in blood vessels was reported by (Gimenez et al., 1997). They indicated that equol induced a modest vasorelaxation with was 10-fold more potent than furosemide on precontracted isolated rat aortic rings. As many metabolic studies have found, daidzein is metabolized by intestinal bacteria to equol, which has a higher estrogenic potency than pure isoflavones (Dixon, 2004; Beck et al., 2005). Equol was also detected in plasma and

urine of sheep after intraruminal infusion of formononetin (Braden et al., 1967), as well as in sheep grazing clover (Shutt and Braden, 1968).

Chin-Dusting et al. (2001), using rat isolated aortic rings, reported  $17\beta$ -oestradiol, equol, and four other isoflavone metabolites, dihydrodaidzein, cis-tetrahydrodaidzein, trans-tetrahydrodaidzein, and dehydroequol all significantly antagonized contractile responses to noradrenaline. What's more, dose-dependent vasodilatory responses were observed in noradrenaline precontracted rat aorta by  $17\beta$ -oestradiol and five other isoflavone metabolites (Chin-Dusting et al., 2001).

Equol also showed vasorelaxant activity in rat carotid arteries in vitro and rat basilar arteries in vivo as a potent compound similar with daidzein (Jackman et al., 2007). This finding was consistent with the vasorelaxant effect of daidzein found in rabbit basilar (Torregrosa et al., 2003) and rat mesenteric arteries (Nevala et al., 1998). Other isoflavones, formononetin and biochanin A were also reported to relax contracted rat aortic rings induced by phenylephrine in a dose-dependent manner (Wu et al., 2010).

An in vivo study by Chin-Dusting et al. (2004) was the first report of a direct dilatory effect of an isoflavone metabolite in humans. They demonstrated that dehydroequol caused a significant vascular dilation in the forearm resistance arteries. Likewise, brachial artery administration of the isoflavone genistein was reported to increase the blood flow of forearm resistance artery, which was interpreted as an outcome of vasodilation (Walker et al., 2001). However, the exact mechanisms of isoflavone and their metabolites as vasodilators have not been fully elucidated.



Substantial human, animal, and cell/tissue culture studies have indicated that isoflavones and their metabolites induced vasodilation was mediated by endothelium. The endothelium of the vascular system plays important roles on regulating blood flow, vascular tone, vascular smooth muscle growth, inflammation, coagulation and fibrinolysis (Hall et al., 2005). The release of nitric oxide (NO) mainly triggers the endothelium-dependent vasodilation, which relaxes the smooth muscle and dilates the vessel. In endothelium, NO is produced by endothelial NO synthase (eNOS) (Hall et al., 2005). In vitro evidence was provided by several animal trials, which found genistein, daidzein and isoflavone metabolites induced vasodilative response was endothelium dependent on rat aortic rings (Chin-Dusting et al., 2001), pulmonary arteries (Karamsetty et al., 2001), and abdominal aorta (Jiang et al., 2003). In vivo studies reported that brachial artery administration of genistein caused vasodilation which was antagonized by eNOS inhibitor L-NMMA (Walker et al., 2001). What's more, both elevated vasodilation and eNOS activity were reported in ovariectomised rats consuming dietary isoflavones (Yamaguchi et al., 2001; Catania et al., 2002). Genomic and non-genomic endoplasmic reticulum-mediated activation of eNOS were believed to be responsible for the stimulation of NO production by isoflavones (Hall et al., 2005).

In contrast, genistein and daidzein were reported to cause vasodilative responses even when endothelium was denuded in rat aortic arteries (Li et al., 2004), and rat mesenteric arteries (Nevala et al., 1998). In addition, Jackman et al. (2007) demonstrated that the vasorelaxation of carotid arteries in vitro and basilar artery in vivo to equol was independent of endothelium and eNOS activity. At present, there is no exact answer as

to whether the vasodilative response to isoflavone and its metabolites is wholly endothelium dependent or independent, and the mechanism of isolated isoflavones varies among different tissue types of different animal models.

Interestingly, an in vitro study indicated that genistein-induced vascular relaxation of rabbit femoral arteries was partially endothelium-dependent, and a calcium antagonistic mechanism was involved after the removal of endothelium (Ji et al., 2002). Similarly, Wu et al. (2010) suggested that formononetin caused vasorelaxation in rat aortic arteries via both endothelium dependent and independent pathways. They reported that the activation of large conductance  $\text{Ca}^{2+}$ -dependent  $\text{K}^+$  ( $\text{BK}_{\text{Ca}}$ ) channels and ATP-sensitive  $\text{K}^+$  channel ( $\text{K}_{\text{ATP}}$ ) was involved in endothelium independent mechanism. Again, more evidences are necessary to better understand the molecular mechanisms of isoflavones as a vasodilator.

## Conclusion

### Interaction of Ergot Alkaloids and Isoflavones

Implantation of steroid hormones have been shown to increase body weight gain of calves grazing endophyte-infested tall fescue (Davenport et al., 1993; Bransby et al., 1994; Coffey et al., 2001; Aiken et al., 2006). What's more, growth performance studies have reported that addition of clover to infected tall fescue increased steer gain significantly (Thompson et al., 1993). The dilution of toxic tall fescue by clover was thought the reason for the positive impacts on performance. As mentioned in the last chapter, red clover contains a variety of isoflavones, such as genistein, daidzein, biochanin A and

formononetin (Beck et al., 2005). It is possible that the isoflavone and ergot alkaloid interaction was part of the reason to cause impact in performance mentioned above. However, more information about the interaction of ergot alkaloids and isoflavones are necessary, especially in terms of its potentially positive effects on fescue toxicosis.

### Objectives

Substantial evidence has shown that ergot alkaloids and isoflavones are able to elicit opposing physiological activities on the vasculature. Ergot alkaloids share some structural similarities with biogenic amines (i.e. dopamine, epinephrine, norepinephrine, serotonin) and thus can induce vasoconstriction by binding biogenic amine receptors. On the other hand, isoflavones have estrogenic activities and might be able to cause endothelium dependent or independent vasorelaxation. Although the exact mechanisms of their vascular bioactivities have not been fully defined, current knowledge indicates that these vascular reactions are triggered by different mechanisms. Nevala et al. (1998) reported that isoflavones relax noradrenaline pre-contracted rat mesenteric arteries. Likewise, Egert et al. (2014) demonstrated that ergot alkaloids were vasoactive in bovine mesenteric vasculature, as well as dietary exposure to ergot alkaloids decreased the contractility of mesenteric vasculature. Thus, it is hypothesized that isoflavones may attenuate ergot alkaloid induced vasoconstriction and further alleviate the diminished contractility of mesenteric vasculature after pre-exposure to ergot alkaloids. The objective of this study is to investigate the interaction of isoflavones and ergot alkaloids on vasoactivity of bovine mesenteric vasculature and to determine if pre-incubation with formononetin, biochanin A, or ergovaline-containing tall fescue seed extract and their

combinations affect the ergotamine-induced contractility of bovine mesentery vasculature.

Table 1.1 Substituents of ergopeptide (Hafner et al., 2008).

Toxin	Toxin group	Substituent R1	Substituent R2
Ergocomine	Ergopeptide	CH(CH <sub>3</sub> ) <sub>2</sub>	CH(CH <sub>3</sub> ) <sub>2</sub>
Ergocristine	Ergopeptide	CH <sub>2</sub> C <sub>6</sub> H <sub>5</sub>	CH(CH <sub>3</sub> ) <sub>2</sub>
Ergotamine	Ergopeptide	CH <sub>2</sub> C <sub>6</sub> H <sub>5</sub>	CH <sub>3</sub>
Ergosine	Ergopeptide	CH(CH <sub>3</sub> )C <sub>2</sub> H <sub>5</sub>	CH <sub>3</sub>
α-Ergocryptine	Ergopeptide	CH(CH <sub>3</sub> ) <sub>2</sub>	CH <sub>3</sub> CHCH <sub>2</sub> CH <sub>3</sub>

Table 1.2 Ergot alkaloids and their biogenic amine receptors.

Ergot Alkaloid	Animal	Vessel type	Adrenergic receptor class	Serotonergic receptor class	Citation
Ergovaline	Cow	Uterine and umbilical artery	Not by $\alpha_1$ -adrenergic receptor	5HT <sub>2A</sub>	Dyer (1993)
	Rat	Tail artery	ND	5HT <sub>2A</sub>	Schoning et al. (2001)
		Thoracic artery	$\alpha_1$ -adrenergic receptor	ND	
	Guinea pig	Iliac artery	ND	5HT <sub>1B/1D</sub>	
Ergotamine	Rat	Tail artery	ND	5HT <sub>2A</sub>	
Ergine (Lysergamide)	Cattle	Lateral saphenous vein	$\alpha_1$ -adrenergic receptor	5HT <sub>2A</sub>	Oliver et al. (1993)
			$\alpha_2$ -adrenergic receptor	Not 5HT <sub>1</sub>	
Endophyte-infested tall fescue	Cattle	Lateral saphenous vein	$\alpha_2$ -adrenergic receptor	ND	Oliver et al. (1998)
			ND	5HT <sub>2A</sub>	Klotz et al. (2012)
				5HT <sub>7</sub>	
				5HT <sub>2A</sub>	Klotz et al. (2013)

Figure 1.1 Ergoline ring system.(Garner et al., 1993)

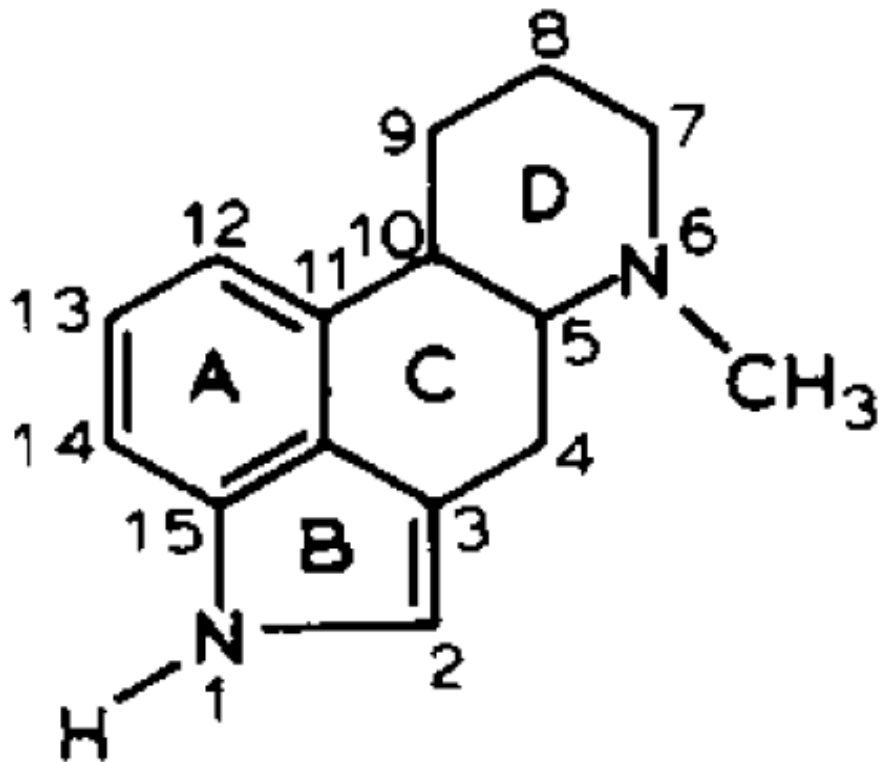
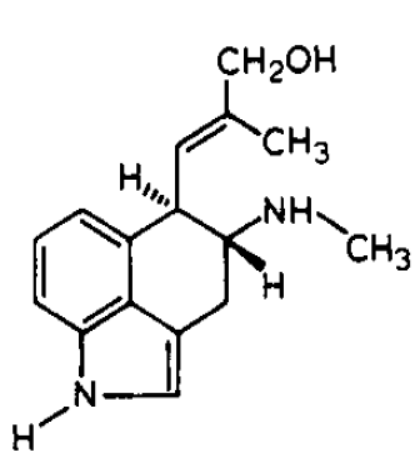
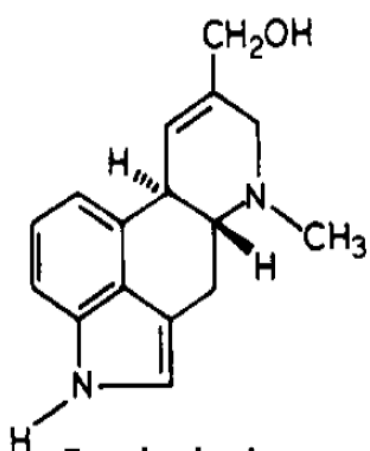


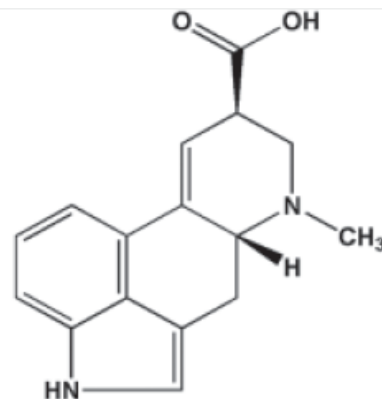
Figure 1.2 Chemical structures of the common clavine alkaloids, lysergic acid derivatives, and ergopeptine alkaloids.



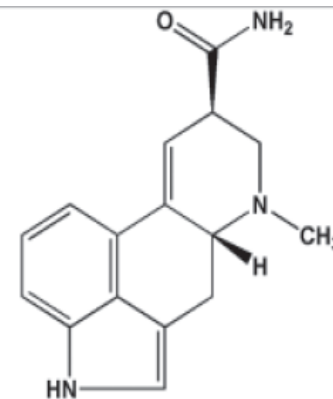
**Chanoclavine**



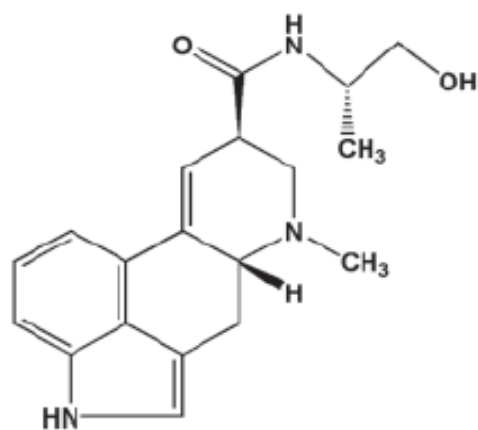
**Emyloclavine**



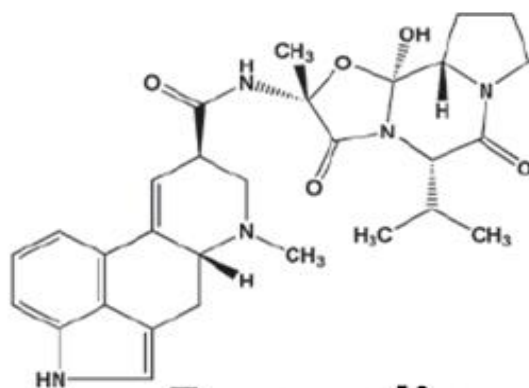
**Lysergic Acid**



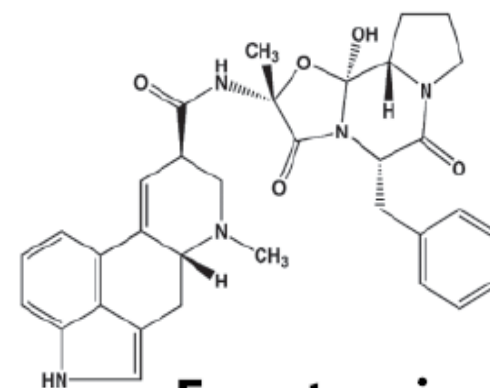
**Lysergic Acid Amide**



**Ergonovine**



**Ergovaline**



**Ergotamine**



Figure 1.3 General structure of ergopeptine alkaloids. (Hafner et al., 2008)

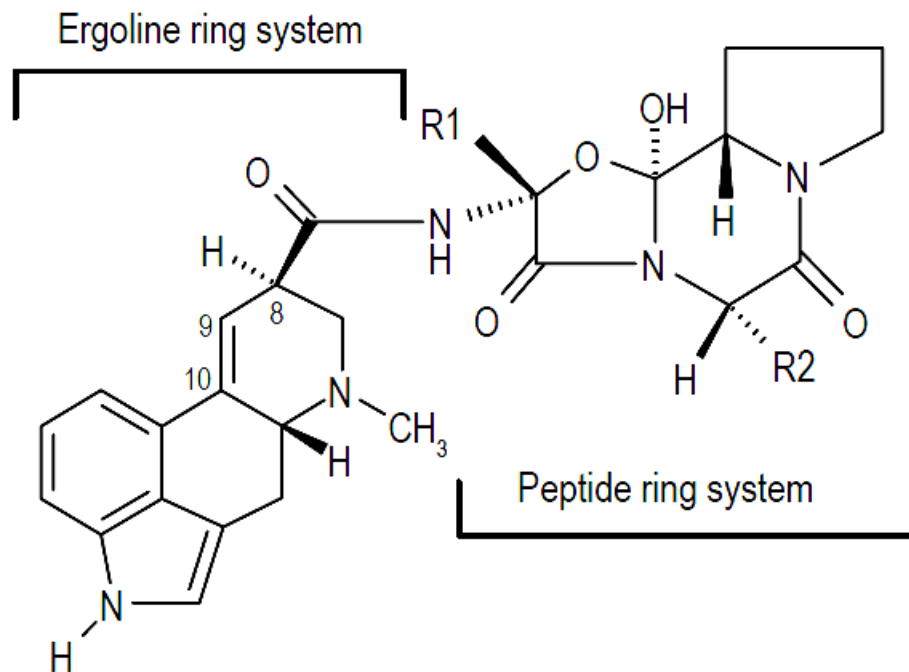
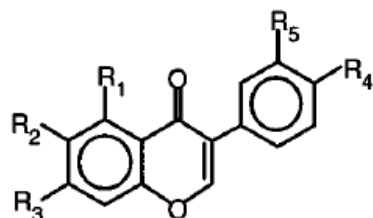


Figure 1.4 Chemical structures of the isoflavones found in legumes. (Kurzer and Xu, 1997)



Isoflavone	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	R <sub>4</sub>	R <sub>5</sub>
Daidzein	H	H	OH	OH	H
Genistein	OH	H	OH	OH	H
Glycitein	H	OCH <sub>3</sub>	OH	OH	H
Daidzin	H	H	O-glucoside	OH	H
Genistin	OH	H	O-glucoside	OH	H
Glycitin	H	OCH <sub>3</sub>	O-glucoside	OH	H
Formononetin	H	H	OH	OCH <sub>3</sub>	H
Biochanin A	OH	H	OH	OCH <sub>3</sub>	H

## Chapter 2. Materials and Methods

No live animals were involved this study, so approval from the University of Kentucky Animal Care and Use Committee was not required.

### Animals and Tissue Collection

Five Angus heifers (BW =  $639 \pm 39$  kg) were slaughtered and tissues were collected at the University of Kentucky abattoir. After the gastrointestinal tract was removed from the carcass, the cecum, ileocecal fold, and the ileal flange were identified as landmarks. Within the mesentery supporting the ileal flange, multiple branches of the mesenteric artery and vein bundles were dissected out and submerged in oxygenated Krebs-Henseleit buffer (95% O<sub>2</sub>/5% CO<sub>2</sub>; pH= 7.4; 11.1 mM D-glucose; 1.2 mM MgSO<sub>4</sub>; 1.2 mM KH<sub>2</sub>PO<sub>4</sub>; 4.7 mM KCl; 118.1 mM NaCl; 3.4 mM CaCl<sub>2</sub>; 24.9 mM NaHCO<sub>3</sub>; Sigma Chemical Co., St. Louis, MO) for transport to the laboratory. Samples were placed on ice until cleaned. At the time of cleaning, surrounding fat and connective tissues were carefully dissected away, and mesentery artery and vein were separated under a magnifying lamp (2.5 to 5.0X magnification). Cleaned vessels were sliced into 2-mm segments and examined under a dissecting scope (Semi 2000-C, Carl Zeiss Inc., Oberkochen, Germany) at 12.5X magnification to ensure the usability of the vessels. Cross-sections with abnormalities (branches, valves, or structural damage) were replaced with structurally integral ones.

### Pre-myograph Incubations

A tall fescue seed extract was prepared as described by Foote et al. (2012) to contain a  $1 \times 10^{-6}$  M working concentration of ergovaline. Duplicates of each vessel type were incubated in tissue culture flasks with a 50-mL volume of Krebs-Henseleit buffer containing: only buffer (**Control**);  $1 \times 10^{-6}$  M ergovaline-containing tall fescue seed extract (**EXT**);  $1 \times 10^{-6}$  M formononetin (**F**;  $\geq 99.0\%$ ; 47752-5MG-F; Sigma Chemical Co., St. Louis, MO); or  $1 \times 10^{-6}$  M biochanin A (**B**; D2016; Sigma Chemical Co., St. Louis, MO); and combinations of  $1 \times 10^{-6}$  M EXT and  $1 \times 10^{-6}$  M F (**EXT + F**);  $1 \times 10^{-6}$  M EXT and  $1 \times 10^{-6}$  M B (**EXT + B**);  $1 \times 10^{-6}$  M F and  $1 \times 10^{-6}$  M B (**F + B**); or  $1 \times 10^{-6}$  M EXT and  $1 \times 10^{-6}$  M F and  $1 \times 10^{-6}$  M B (**EXT + F + B**). All buffer solutions were pre-warmed for 30 min in a CO<sub>2</sub> incubator (95% O<sub>2</sub>/5% CO<sub>2</sub>; 37 °C; Nu-8500, NUAIRE, Inc. Plymouth, MN) prior to blood vessel addition. Duplicate blood vessel segments were randomly placed into each treatment flask and incubated in the same conditions for 2 h. Immediately after the 2-h incubation, dimensional measurements of cross-sections were recorded only for mesentery artery using Axiovision (version 20, Carl Zeiss, Inc.).

### Experimental Myograph Protocol

Following the incubations, an ergotamine concentration response experiment was conducted using the procedures described by Egert et al. (2014) and Klotz (2014). Ergotamine (ergotamine D-tartrate; 97%; 45510; Aldrich, Milwaukee, WI) standards were prepared by diluting stock solutions (0.0201 M) with dimethyl sulfoxide to working

concentrations that resulted in a concentration range of  $5 \times 10^{-9}$  to  $1 \times 10^{-4}$  M in the myograph chamber (contained 5 mL of Krebs-Henseleit buffer).

Blood vessel cross-sections were gently mounted on the myograph (Multichamber myograph; DMT 610M, Danish Myo Technology, Atlanta, GA) by inserting the supports through the lumen in individual myograph chambers containing 5 mL modified Krebs-Henseleit buffer and continuously gassing (95% O<sub>2</sub>/5% CO<sub>2</sub>; pH = 7.4; 37°C). The incubation buffer was modified from transport Krebs-Henseleit buffer by adding desipramine ( $3 \times 10^{-5}$  M; D3900; Sigma Chemical Co.) to inhibit the reuptake mechanisms of biogenic amines and propranolol ( $1 \times 10^{-6}$  M; P0844; Sigma Chemical Co.) to block the non-specific binding to  $\beta$ -adrenergic receptors. A 90-min equilibration period with buffer replacement occurring every 15 min was completed based on the conditions above to achieve a stable resting tension of approximately 1 g. After the completion of equilibration, 500  $\mu$ L of a 1.32 M KCl (Sigma Chemical Co.) solution, resulting in a 120 mM KCl in a myograph chamber, was added and incubated for 15 min to evaluate tissue viability and to be used to normalize treatment data. Incubation buffer was replaced every 15 min until vessel tension returned to the 1 g baseline. Once the vessel returned to baseline, addition of ergotamine standards were initiated in order of increasing concentration. Ergotamine additions were added in 15-min intervals consisting of a 9-min incubation period, two 2.5-min buffer washes, and a third, final buffer replacement that was followed by 1-min recovery before the next ergotamine addition. This 15 min cycle was repeated for the rest of the 9 remaining ergotamine additions. Following the 1-min

recovery after the final ergotamine addition, vessels were again exposed to 120 mM KCl to confirm the vessel viability at the end of the experiment.

#### Data Collection and Statistical Analysis

The isometric contractions of the different pre-incubated mesenteric vessels to KCl and ergotamine additions were digitized and recorded as grams of tension using a PowerLab/8sp and Chart software (version 7.3, ADInstruments, Colorado Springs, Co.). Baseline tension was measured immediately before the addition of 1.32 M KCl. For all contractile response data, the maximum observed tension (g) in the 9 min-incubation period was recorded as the contractile response. Contractile response data were corrected for baseline tension (subtraction of the baseline measurement) and normalized as a percentage of the reference compound KCl induced maximum contractile response (grams of tension). The differences of tissue response due to the variations of vessel size and individual animal were taken into account by this data normalization. The contractile response to ergotamine was determined and presented as percentage means  $\pm$  SEM. A measurement of potency, or the half maximal effective concentration ( $EC_{50}$ ) was calculated by GraphPad Prism software 5 (GraphPad Software Inc., La Jolla, CA) using a nonlinear regression with fixed slope. The sigmoidal concentration response curves of pre-treated mesentery artery and vein to ergotamine was plotted by using a 3-parameter equation:

$$y = \text{bottom} + \frac{(\text{top} - \text{bottom})}{[1 + 10^{(\log EC_{50} - x)}]},$$

where top and bottom are the plateaus of contractile response as percentage of 120 mM KCl maximum response.  $EC_{50}$  is the molar concentration of ergotamine inducing 50% of the KCl maximum response.

All data were analyzed using the MIXED model of SAS (SAS 9.4, SAS Inst. Inc., Cary, NC). Contractile response data of mesentery artery and vein were analyzed separately as two groups: treatments with and without EXT. Data for contractile response (within each ergotamine concentration), KCl maximal response, inside and outside diameters for mesenteric artery (data could not be obtained for mesenteric vein samples) were analyzed as a completely randomized design with a factorial treatment arrangement. The fixed effects included the effects of F, B, and the interaction of  $F \times B$  in the presence or absence of EXT. Due to the shape of the response curve,  $EC_{50}$  data were analyzed only in treatments without EXT from mesentery artery using a completely randomized design with treatment as fixed variable. For all data, an ANOVA was conducted and pair-wise comparisons of least square means ( $\pm$  SEM) were performed if the probability of a greater F-statistic was significant for the tested effect and interaction. Mean separation was performed with the LSD feature of SAS. Differences are denoted as significant at  $P < 0.05$ , unless specifically reported otherwise.

### Chapter 3. Results

The F and B incubation pre-treatment did not impact ( $P > 0.05$ ) the maximum contractile response of mesenteric artery or mesenteric vein to 120 mM KCl either without EXT (Table 3.1) or with EXT (Table 3.2). The pre-treatments with EXT did not

compromise the vessel viability, which was indicated by the ending response to KCl for either the artery or vein (Figure 3.1). In mesenteric artery, the inside and outside diameter after the 2 h incubation were not affected ( $P > 0.05$ ) by F or B in both treatments groups with or without EXT (Table 3.1, Table 3.2). However, there was a tendency ( $P < 0.1$ ) for F treated vessels to have a larger inside diameter for those pretreated with EXT, and a smaller outside diameter for those pretreated without EXT (Table 3.2).

In the mesenteric artery, ergotamine induced similar contractile responses in all treatments (Figure 3.2a) with  $-\log EC_{50}$  values ( $5.99 \pm 0.14 M$ ,  $5.80 \pm 0.14 M$ ,  $5.88 \pm 0.14 M$ ,  $5.74 \pm 0.14 M$ , respectively) that did not differ with each other ( $P = 0.6338$ ). Within each ergotamine concentration, tendencies for F  $\times$  B interactions in mesenteric artery were observed at ergotamine  $5 \times 10^{-9} M$ ,  $1 \times 10^{-8} M$ ,  $5 \times 10^{-8} M$  ( $P = 0.0932$ ,  $P = 0.0703$ ,  $P = 0.0840$ , respectively; Table 3.3) for treatments without EXT. Contractile responses of F + B treated mesenteric artery were greater than B at ergotamine concentrations  $1 \times 10^{-8} M$  and  $5 \times 10^{-8} M$  ( $P < 0.05$ ). In mesenteric vein, ergotamine induced contractile responses reached the maximum response at  $1 \times 10^{-6} M$  for Control, B, F, F + B treatments (Figure 3.2b), and then declined to negative values with increases in ergotamine concentration.

For the blood vessels incubated with EXT the contractile responses decreased as the concentration of ergotamine increased in both mesenteric artery and mesenteric vein (Figure 3.2c, 3.2d). In mesenteric artery, a main effect of B was observed at ergotamine concentrations of  $1 \times 10^{-7} M$  and  $5 \times 10^{-7} M$  (Table 3.4), with treatments that contained B having higher contractile responses ( $P < 0.05$ ). Also, treatments containing B tended ( $P <$



0.1) to have greater responses than treatments without B at ergotamine concentrations of  $1 \times 10^{-6} M$ ,  $5 \times 10^{-6} M$ , and  $5 \times 10^{-5} M$  (Table 3.4).

In the mesenteric vein, the contractile response of all treatments with EXT to ergotamine decreased and remained below zero from the second ergotamine addition ( $1 \times 10^{-8} M$ ) to the last addition at  $1 \times 10^{-4} M$ . Tendencies for main effects of B ( $P = 0.06$ ) and a F  $\times$  B interaction ( $P = 0.09$ ) were observed at ergotamine concentrations of  $5 \times 10^{-9} M$ , where EXT treatment had a lower contractile response than EXT + B ( $P < 0.05$ ; Table 3.4). Treatments containing F had greater contractile responses to ergotamine at  $1 \times 10^{-5} M$ ,  $5 \times 10^{-5} M$ , and  $1 \times 10^{-4} M$  in mesenteric vein (main effect of F;  $P < 0.05$ ; Table 3.4). Within ergotamine concentrations, at  $5 \times 10^{-5} M$  the contractile response of EXT was the lowest ( $P < 0.05$ ).

Table 3.1 Inside diameter, outside diameter and SEM of mesenteric artery, and the mean KCL maximum response of mesenteric artery and vein to pre-treatment without tall fescue extract: only Krebs-Henseleit buffer (**Control**);  $1 \times 10^{-6}$  M formononetin (**F**); or  $1 \times 10^{-6}$  M biochanin A (**B**); and combination of  $1 \times 10^{-6}$  M F and  $1 \times 10^{-6}$  M B (**F + B**)

Item	Control	F	B	F + B	SEM	P-value <sup>1</sup>		
						F	B	F x B
Mesenteric artery								
KCL maximum response, g	4.36	4.42	4.22	3.66	0.46	0.5837	0.3319	0.4951
Inside diameter, mm	0.81	0.79	0.86	0.77	0.06	0.3774	0.7714	0.6142
Outside diameter, mm	1.72	1.64	1.75	1.57	0.07	0.0901	0.7440	0.4721
Mesenteric vein								
KCL maximum response, g	1.70	1.81	1.89	2.22	0.21	0.3115	0.1784	0.6160

<sup>1</sup> F, main effect of formononetin; B, main effect of biochanin A; F x B, interaction of formononetin and biochanin A.

Table 3.2 Inside diameter, outside diameter and SEM of mesenteric artery, and the mean KCL maximum response of mesenteric artery and vein to pre-treatment with tall fescue seed extract:  $1 \times 10^{-6}$  M ergovaline-containing tall fescue seed extract (**EXT**); combinations of  $1 \times 10^{-6}$  M EXT and  $1 \times 10^{-6}$  M F (**EXT + F**);  $1 \times 10^{-6}$  M EXT and  $1 \times 10^{-6}$  M B (**EXT + B**); or  $1 \times 10^{-6}$  M EXT and  $1 \times 10^{-6}$  M F and  $1 \times 10^{-6}$  M B (**EXT + F + B**).

Item	EXT	EXT + F	EXT + B	EXT + F + B	SEM	P-value <sup>1</sup>		
						F	B	F x B
Mesenteric artery								
KCL maximum response, g	3.43	2.68	3.08	2.84	0.37	0.1887	0.8022	0.5010
Inside diameter, mm	0.72	0.78	0.60	0.72	0.05	0.0742	0.0649	0.5034
Outside diameter, mm	1.64	1.57	1.51	1.59	0.07	0.9189	0.4256	0.2855
Mesenteric vein								
KCL maximum response, g	1.90	2.20	2.09	2.49	0.21	0.1110	0.2646	0.8146

<sup>1</sup> F, main effect of formononetin; B, main effect of biochanin A; F x B, interaction of formononetin and biochanin A.

Table 3.3 The analysis of variance and *P*-values of main effect of formononetin (F), biochanin A (B), and the interaction of formononetin and biochanin A (F x B) for pre-treatments without tall fescue extract: only Krebs-Henseleit buffer;  $1 \times 10^{-6}$  M formononetin; or  $1 \times 10^{-6}$  M biochanin A; and combination of  $1 \times 10^{-6}$  M formononetin and  $1 \times 10^{-6}$  M biochanin A on every ergotamine concentration.

Ergotamine Concentration, M	<i>P</i> -value <sup>1</sup>		
	F	B	F x B
<i>Mesenteric artery</i>			
$5 \times 10^{-9}$	0.2281	0.9117	0.0932
$1 \times 10^{-8}$	0.1446	0.2723	0.0703
$5 \times 10^{-8}$	0.1963	0.5085	0.0840
$1 \times 10^{-7}$	0.2943	0.8303	0.3059
$5 \times 10^{-7}$	0.8788	0.7897	0.4319
$1 \times 10^{-6}$	0.6784	0.7843	0.6378
$5 \times 10^{-6}$	0.2674	0.9888	0.7172
$1 \times 10^{-5}$	0.3790	0.9720	0.9700
$5 \times 10^{-5}$	0.4322	0.8098	0.7747
$1 \times 10^{-4}$	0.6140	0.5352	0.5949
<i>Mesenteric vein</i>			
$5 \times 10^{-9}$	0.9175	0.2667	0.8545
$1 \times 10^{-8}$	0.9963	0.9648	0.5015
$5 \times 10^{-8}$	0.6737	0.6700	0.2827
$1 \times 10^{-7}$	0.7952	0.6422	0.4082
$5 \times 10^{-7}$	0.9871	0.4340	0.7427
$1 \times 10^{-6}$	0.9304	0.2983	0.7009
$5 \times 10^{-6}$	0.7795	0.4265	0.6996
$1 \times 10^{-5}$	0.5048	0.7964	0.6229
$5 \times 10^{-5}$	0.2860	0.9309	0.6051
$1 \times 10^{-4}$	0.2286	0.8638	0.8886

<sup>1</sup> F, main effect of formononetin; B, main effect of biochanin A; F x B, interaction of formononetin and biochanin A.

Table 3.4 The analysis of variance and *P*-values of main effect of formononetin (F), biochanin A (B), and the interaction of formononetin and biochanin A (F x B) for pre-treatments with tall fescue seed extract:  $1 \times 10^{-6}$  M ergovaline-containing tall fescue seed extract; combinations of  $1 \times 10^{-6}$  M ergovaline-containing tall fescue seed extract and  $1 \times 10^{-6}$  M formononetin;  $1 \times 10^{-6}$  M ergovaline-containing tall fescue seed extract and  $1 \times 10^{-6}$  M biochanin A; or  $1 \times 10^{-6}$  M ergovaline-containing tall fescue seed extract and  $1 \times 10^{-6}$  M formononetin and  $1 \times 10^{-6}$  M biochanin A on every ergotamine concentration.

Ergotamine Concentration, M	P-value <sup>1</sup>		
	F	B	F x B
<i>Mesenteric artery</i>			
$5 \times 10^{-9}$	0.9418	0.7127	0.6984
$1 \times 10^{-8}$	0.4289	0.3081	0.9555
$5 \times 10^{-8}$	0.2200	0.1114	0.7707
$1 \times 10^{-7}$	0.2009	0.0438	0.7342
$5 \times 10^{-7}$	0.2482	0.0302	0.5680
$1 \times 10^{-6}$	0.2109	0.0572	0.5798
$5 \times 10^{-6}$	0.2131	0.0863	0.5240
$1 \times 10^{-5}$	0.3070	0.1269	0.6992
$5 \times 10^{-5}$	0.2432	0.0975	0.8691
$1 \times 10^{-4}$	0.1788	0.1170	0.7737
<i>Mesenteric vein</i>			
$5 \times 10^{-9}$	0.7453	0.0694	0.0976
$1 \times 10^{-8}$	0.9145	0.7666	0.4060
$5 \times 10^{-8}$	0.4529	0.9824	0.5007
$1 \times 10^{-7}$	0.1753	0.8547	0.3217
$5 \times 10^{-7}$	0.1483	0.4810	0.1878
$1 \times 10^{-6}$	0.1872	0.5756	0.3217
$5 \times 10^{-6}$	0.1566	0.4390	0.2674
$1 \times 10^{-5}$	0.0339	0.3013	0.1160
$5 \times 10^{-5}$	0.0212	0.1711	0.0794
$1 \times 10^{-4}$	0.0059	0.2382	0.1359

<sup>1</sup> F, main effect of formononetin; B, main effect of biochanin A; F x B, interaction of formononetin and biochanin A.

Figure 3.1 Example of a typical response of mesenteric artery (a) and vein (b) cross sections, after pre-treated with  $1 \times 10^{-6} M$  EXT and  $1 \times 10^{-6} M$  F and  $1 \times 10^{-6} M$  B (EXT + F + B), to increasing concentrations of ergotamine ( $5 \times 10^{-9}$  to  $1 \times 10^{-4} M$ ). The rectangles highlighted region are the initial and end KCl ( $0.12 M$ ) additions.

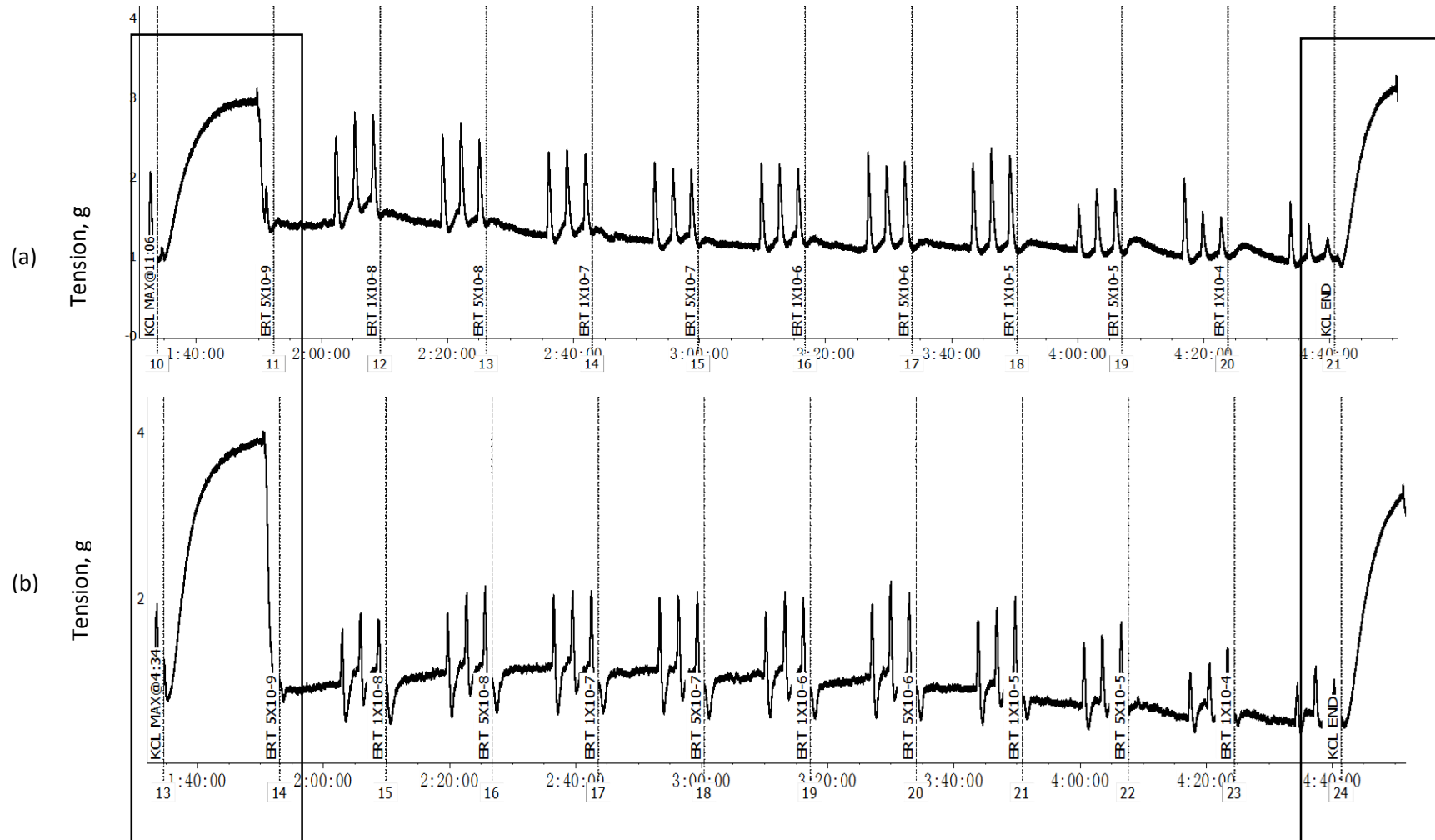
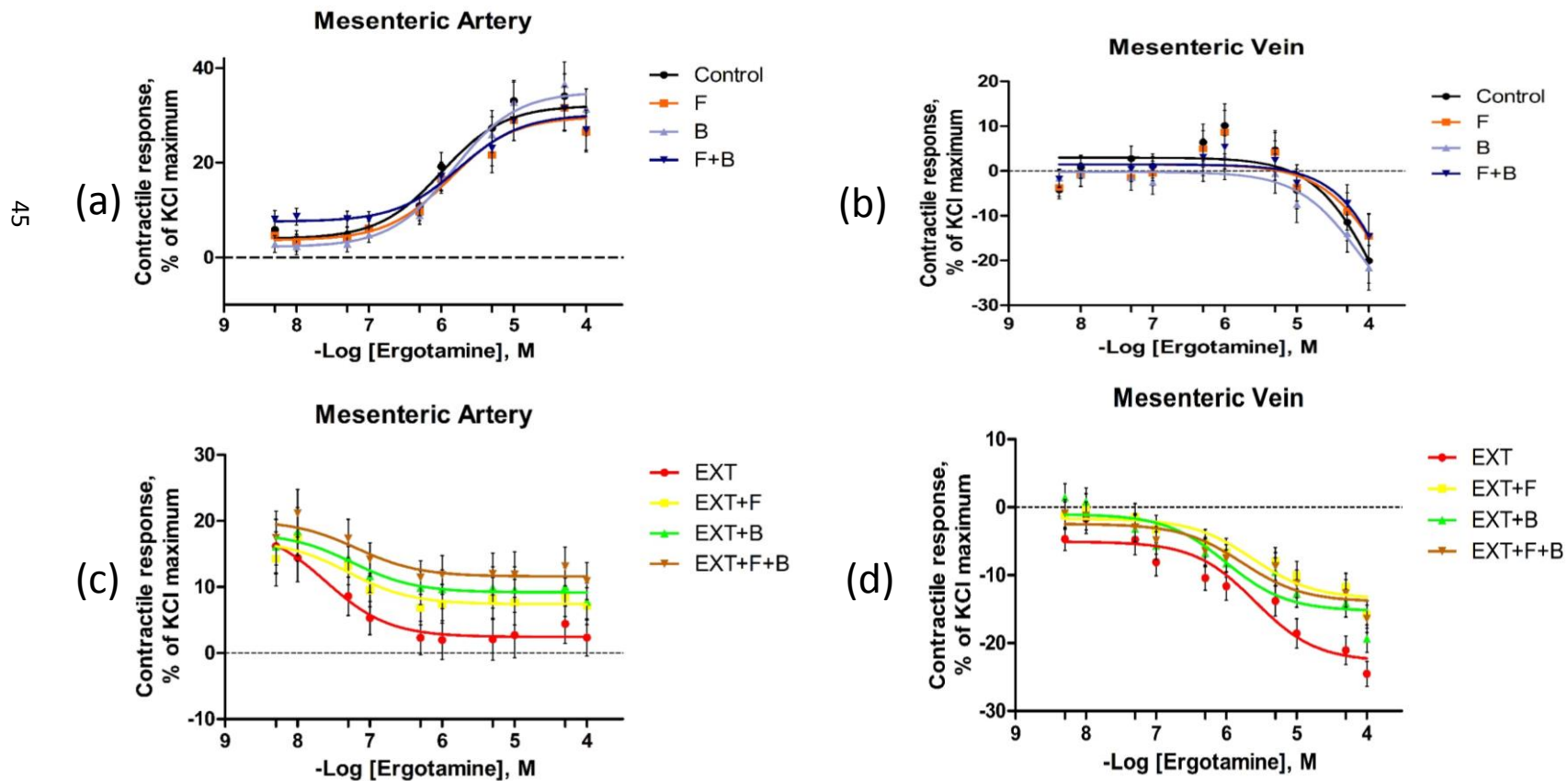


Figure 3.2 Mean contractile response, as % KCl maximum of mesenteric artery (a, c) and vein (b, d) to increasing concentrations of ergotamine for two groups of pre-treatments, without tall fescue seed extract: only Krebs-Henseleit buffer (**Control**);  $1 \times 10^{-6}$  M formononetin (**F**); or  $1 \times 10^{-6}$  M biochanin A (**B**); and combination of  $1 \times 10^{-6}$  M F and  $1 \times 10^{-6}$  M B (**F + B**) (a, b); with tall fescue seed extract:  $1 \times 10^{-6}$  M ergovaline-containing tall fescue seed extract (**EXT**); combinations of  $1 \times 10^{-6}$  M EXT and  $1 \times 10^{-6}$  M F (**EXT + F**);  $1 \times 10^{-6}$  M EXT and  $1 \times 10^{-6}$  M B (**EXT + B**); or  $1 \times 10^{-6}$  M EXT and  $1 \times 10^{-6}$  M F and  $1 \times 10^{-6}$  M B (**EXT + F + B**) (c, d). The regression lines were plotted of each treatment using a nonlinear regression with fixed slope and the sigmoidal concentration response curves were calculated by the following equation:  $y = \text{bottom} + [(\text{top} - \text{bottom}) / (1 + 10^{(\log EC_{50} - x)})]$ , where top and bottom are the plateaus of contractile response as percentage of 120 mM KCl maximum response.  $EC_{50}$  is the molar concentration of ergotamine inducing 50% of the KCl maximum response.



## Chapter 4. Discussion and Conclusion

To our knowledge, this study was the first to investigate the interaction of ergoalkaloids and isoflavones on in vitro mesenteric vasculature. Physiological effects of isoflavones and their metabolites on vasculature have been extensively studied on different vessel types in humans (Walker et al., 2001; Chin-Dusting et al., 2004), and rat models (Gimenez et al., 1997; Chin-Dusting et al., 2001; Jackman et al., 2007; Wu et al., 2010). However, to date, isoflavones' vasoactivity have not been studied in bovine mesenteric vasculature.

Several studies have demonstrated that cattle grazing endophyte-infected tall fescue had reduced contractile responses to 5HT and as well as ergot alkaloids in the lateral saphenous vein (Klotz et al., 2012; Klotz et al., 2013). Recently, a study using mesenteric artery and vein from steers that had been intraruminally dosed with endophyte-infected tall fescue seed observed a decreased or absent constrictive response to ergot alkaloids (Egert et al., 2014). In the current study, an in vitro pre-myograph incubation of bovine mesenteric vasculature in the medium containing endophyte-infected tall fescue seed extract was used to achieve an ergot alkaloid pre-treatment. The endophyte-infected tall fescue seed extract (Foote et al., 2012) and isoflavones (formononetin and biochanin A) were added to the myograph medium at equimolar final concentrations of  $1 \times 10^{-6} M$ . It has been reported that formononetin and biochanin A both induced vasorelaxation in phenylephrine precontracted rat isolated thoracic aorta at  $1 \times 10^{-6} M$  (Wu et al., 2010). However, the  $1 \times 10^{-6} M$  ergovaline in the



endophyte-infected tall fescue seed extract may be considered a high dose compared to the physiological level encountered by cattle grazing endophyte-infected tall fescue. Nevertheless, the viability of mesenteric artery and vein were not compromised by these amounts of ergot alkaloids which was supported by the fact that they were both active to the ending KCl addition.

Studies have reported that many isoflavones and their metabolites can reduce the vasoconstriction induced by KCl in several different vessel types using rat models (Jiang et al., 1991; Jackman et al., 2007). The contractile response to KCl of endothelium denuded rat aortic rings was inhibited by pretreatment with genistein or daidzein at both 30 and 100  $\mu$ M for 30 min (Seok et al., 2008). However, in the same study, pretreatment with genistein or daidzein at 10  $\mu$ M did not induce the vasorelaxation caused by KCl. The similar dose-dependent inhibition of formononetin (10, 30, and 100  $\mu$ M) on the contractile response to KCl was observed in rat mesenteric arteries without endothelium (Sun et al., 2011), where formononetin again failed to inhibit KCl-initiated contraction at 10  $\mu$ M. However, in endothelium-intact rat aortic rings, Zhao et al. (2012) reported that pretreatment with 10, 30, and 50  $\mu$ M of formononetin all significantly inhibited the contractile response to KCl in a noncompetitive manner. In the current study, the prior exposure to formononetin (1  $\mu$ M), biochanin A (1  $\mu$ M), and their combination did not have an impact on maximum contractile response of mesenteric artery and vein to KCl in treatments either with or without EXT. Since previous studies have indicated that the inhibition effects were dose-dependent (Seok et al., 2008; Sun et al., 2011) and noncompetitive (Zhao et al., 2012), it is possible the isoflavone concentration (1  $\mu$ M) in

the current study was not high enough to elicit any inhibitory effects. However, in the current study, from a perspective of reference compounds to normalize the contractile responses, the unaffected maximum KCl response by formononetin and biochanin A were critical.

There is limited information about isoflavones' impact on blood vessel morphology in regards to vessel inside and outside diameters. In the current study, there was no evidence of a formononetin or biochanin A effect on mesenteric artery inside or outside diameter regardless of EXT treatment. However, a tendency of smaller inside diameter induced by formononetin treatment was observed in mesentery artery pre-treated without EXT. One possible explanation for this could be the inhibitory effect of formononetin on vascular smooth muscle cells. Previously, estrogens (tamoxifen (Grainger and Metcalfe, 1996), estradiol (Rosselli et al., 1994)) and phytoestrogens (formononetin and biochanin A (Dubey et al., 1999)) have been shown to inhibit mitogen-induced proliferation, migration and extracellular matrix synthesis of smooth muscle cells. On the other hand, the inside diameter of bovine lateral saphenous veins collected from steers grazing high-endophyte tall fescue pasture were smaller than those from steers grazing low-endophyte mixed-grass pasture (Klotz et al., 2012). Likewise, Egert et al. (2014) observed a smaller outside diameter of bovine mesenteric artery from endophyte-infected tall fescue seed dosed steers than control steers. One possible explanation is the prolonged vasoconstriction induced by ergot alkaloids result in decreased inside or outside diameter. Additionally, the morphological changes of blood vessels, especially the expansion of the smooth muscle layer (tunica media), could also lead to a smaller vessel

insider diameter. It has been observed that calves given ethanolic extracts of tall fescue hay had symptoms of fescue foot and concomitant thickening of vessel walls and smaller vessel lumens in blood vessels of the coronary bands and tail tips (Williams et al., 1975). Similarly, Garner and Cornell (1978) reported a thickening of the smooth muscle layer of peripheral blood vessels after consumption of endophyte-infected tall fescue. Although the exact mechanism associated with thickening of smooth muscle layer is unclear, evidence suggests hyperplasia over hypertrophy. Strickland et al. (1996) reported that ergonovine, ergovaline and  $\alpha$ -ergocryptine stimulate the growth and mitosis of quiescent bovine vascular smooth muscle cells in vitro. Although, in this study, there was no significant impact of formononetin and biochanin A pre-treatment on mesenteric artery inside or outside diameters, tendencies of larger inside diameter caused by formononetin and smaller inside diameter in biochanin A treated mesenteric artery were observed. This controversial observation could not be well explained based on our current knowledge. However, besides vasoconstriction and vasodilation, many other factors could influence the inside or outside diameter, such as vascular smooth muscle hyperplasia and hypertrophy.

In the current study, the ergotamine induced similar contractile response curves in mesenteric artery, and with  $-\log EC_{50}$  values that did not differ among treatments without EXT. The shape of contractile responses were similar with Egert et al. (2014), who reported ergotamine induced contractile responses in mesenteric artery from steers, not exposed to ergot alkaloids, with a  $-\log EC_{50}$  value of  $6.03 \pm 0.4 M$ . Whereas, in mesenteric vein, the shape of contractile response curves were in contrast to the observations of

Egert et al. (2014), which did not drop to negative values after peak. Further, the maximum contractile response of mesenteric vein to ergotamine in the current study was also lower (10% vs. 45% of KCl maximum). It has to be mentioned that the blood vessels used in the previous study (Egert et al., 2014) did not undergo pre-myograph incubation. So, taken together, the 2-hr pre-myograph incubation in the current study seemed to alter the contractile response of mesenteric veins in terms of potency and efficacy to ergotamine.

Substantial evidence has shown many ergot alkaloids are vasoconstrictive in multiple tissues of different animal models (Strickland et al., 2009b; Strickland et al., 2011). Among these vasoactive ergot alkaloids, ergotamine and ergovaline were indicated as more potent vasoconstrictors with relatively lower  $EC_{50}$  values in bovine saphenous vein (Klotz et al., 2007), ruminal vasculature (Foote et al., 2011), and mesenteric vasculature (Egert et al., 2014). The current observation of the contractile response induced by ergotamine was consistent with these previous findings. Even though not completely defined yet, numerous studies have been conducted to investigate the mechanism of ergot alkaloid-caused vasoconstriction. The structural similarities of the ergoline ring system and several biogenic amines (i.e., (nor)epinephrine, serotonin, and dopamine) allows ergot alkaloids to interact with corresponding biogenic amine receptors as ligands (Berde, 1980; Weber, 1980). Substantial evidences have shown that ergot alkaloids interact with dopamine-2 receptor (Larson et al., 1994; Larson et al., 1995),  $\alpha_1$ -adrenergic receptor (Oliver et al., 1993; Schoning et al., 2001),  $\alpha_2$ -adrenergic receptor (Oliver et al., 1998), 5HT<sub>2A</sub> receptor (Dyer, 1993; Klotz et al., 2012; Klotz et al., 2013). The

binding with these G protein-coupled receptors activates the subunit of the heterotrimeric G protein and then triggers various secondary messaging systems and corresponding cytoplasmic signaling transductions.

The pre-myograph incubation with EXT altered the contractile capacity in a manner similar with previous studies. Using a bovine lateral saphenous vein model, Klotz et al. (2012) found DOI (5HT<sub>2A</sub> receptor agonist) induced vessel contractile intensities were 35% lower in high endophyte-infested tall fescue than in low endophyte-infested tall fescue, whereas 5CT (5HT<sub>7</sub> receptor agonist) produced greater (37%) contractile intensities in high endophyte-infested tall fescue. Therefore, they indicated that chronic exposure to ergot alkaloids through grazing endophyte-infested tall fescue altered the vasoconstriction via serotonergic receptors, but in different manners. In a subsequent study (Klotz et al., 2013) they reported a suppression of the contractile response to ergovaline and 5HT in steers grazing Kentucky-31 tall fescue infected with wild-type endophyte. What's more, Egert et al. (2014) showed the similar observation of reduced contractile response to ergotamine in mesenteric vasculature of steers exposed with endophyte-infected tall fescue seed. Taken together, it is possible that the EXT pre-treatment might have reduced the vasoconstriction to ergotamine via altering the potential biogenic amine receptor populations and activities, for example, the antagonistic effects of ergot alkaloids to certain 5HT receptors have been shown previously (Oliver et al., 1993; Pertz and Eich, 1999; Schoning et al., 2001). Although the exact mechanisms are unknown, it is possible these alterations are mediated by the

downregulation of gene transcription associated with secondary messaging pathways of G protein-coupled receptors by ergot alkaloids (Maurer, 1981).

What's more, many in vitro bioassays have proposed, in various vessel types and in many different species, the binding of ergovaline and receptor was irreversible, or the dissociation from the receptor was very slow (Dyer, 1993; Schoning et al., 2001; Klotz et al., 2007; Pesqueira et al., 2014). Likewise, the bioaccumulation effect was reported in bovine lateral saphenous veins after repetitive exposure of ergovaline in vitro (Klotz et al., 2009). These findings may explain the differences of contractile response to initial ergotamine addition ( $5 \times 10^{-9} M$ ) between mesenteric artery incubated with or without endophyte-infected tall fescue seed extract. It is possible that during the two hour pre-incubation with endophyte-infected tall fescue seed extract, the corresponding receptors have been irreversibly bound by ergot alkaloids (mainly  $1 \times 10^{-6} M$  ergovaline) present in the endophyte-infected tall fescue seed extract. This speculation was further validated by the observation that the initial contractile response to ergotamine by EXT (containing  $1 \times 10^{-6} M$  ergovaline) incubated mesenteric artery was numerically similar to  $1 \times 10^{-6} M$  ergotamine induced contractile response of mesenteric artery without EXT incubation. It has been demonstrated that ergotamine and ergovaline are both potent vasoconstrictor inducing similar contractile responses (Klotz et al., 2007).

In the present study, formononetin and biochanin A failed to offset the mesenteric vasculature contraction induced by ergotamine. As discussed earlier in this section, numerous studies have shown the vasodilative effect of isoflavones and their metabolites

in different vessel types. Pretreatment with formononetin (30 and 50  $\mu M$ ) antagonized contractile responses of rat thoracic aortas to norepinephrine in a noncompetitive manner (Zhao et al., 2012). Sun et al. (2011) hypothesized that pre-incubation with formononetin (30 and 100  $\mu M$ ) depressed the contraction of rat mesenteric artery to phenylephrine and 5-HT, and similarly, depression was not observed when formononetin at 10  $\mu M$ . Again, the 1  $\mu M$  formononetin and biochanin A in the present study were possibly not in high enough concentrations to elicit vasorelaxations. Other evidence of the chronic antihypertensive effect of formononetin has been reported based on male spontaneously hypertensive rats (SHR) (Sun et al., 2013). They found that the vasoconstriction of mesenteric artery segments induced by phenylephrine or 5-HT was reduced in formononetin (50 mg/kg per day) orally administrated SHR. What's more, the expression of  $\alpha_1$ -adrenoceptors and 5-HT<sub>2A/1B</sub> receptors in mesenteric artery of formononetin treated SHR decreased (Sun et al., 2013). Even though there might be some species differences between bovine and SHR, the two hour pre-myograph incubation with formononetin or biochanin A in the present study may be not long enough to trigger certain consequences.

With EXT in the pretreatment media, formononetin and biochanin A increased the contractile response of mesenteric artery at several ergotamine concentrations. This has provided the first evidence that the isoflavones, formononetin and biochanin A have potential to alleviate the inhibitory effect of ergot alkaloids. However, current knowledge

from ergot alkaloids and isoflavones are unable to explain this phenomenon, so more investigations on their interaction are needed.

In conclusion, this study indicated that a pre-myograph incubation with formononetin, biochanin A at  $1 \times 10^{-6}$  M and their combination did not affect the contractile response to ergotamine in mesenteric vasculature. The pre-myograph incubation of mesenteric vasculature with endophyte-infected tall fescue seed extract (equivalent to  $1 \times 10^{-6}$  M ergovaline) reduced the vasoactivity of ergotamine. At higher concentrations, formononetin and biochanin A may alleviate this reduction in vasoactivity caused by prior exposure to ergot alkaloids. However, no current data are available to fully support this observation, and future investigations are necessary to understand the mechanism behind the interaction of ergot alkaloids and isoflavone.



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## VITA

### Education

University of Kentucky – Lexington, KY

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M.S Ruminant Nutritional Physiology (in progress)

Advisor: David Harmon Ph.D.

Dissertation: Interaction of isoflavones and endophyte-infected tall fescue seed extract on vasoactivity of bovine mesenteric vasculature.

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B.S. Animal Science

“Outstanding Graduate” by Nanjing Agricultural University

### Professional Experiences

2011.08 – 2012.07      Technician - Production Technology Department. Baotou  
Chuangwei Industrial Co., Ltd.

2010.07 – 2010.09      Intern Technician – Department of Research and Development.  
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### Awards

2007    Excellent Student Leader by Psychology Association of Nanjing Agricultural  
University

2008    First Prize of “Excellent Student Scholarship” by Nanjing Agricultural University

2008    “Best Hall Leader of the Year” by Nanjing Agricultural University

2009    “AoHua Scholarship” by Shenzhen AoHua Agricultural and Animal Husbandry  
Limited Co.

2010    Second Prize of “Excellent Student Scholarship” by Nanjing Agricultural  
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2011    “Outstanding Graduate” by Nanjing Agricultural University

### Publications

Wang, H., Jia, Y., Huang, W., Zheng, Y., & Li, H. (2014). Individual identification of Chinese Holstein Bull by 10 STR loci. *Molecular biology reports*, 41(3), 1201-1206